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Foot-and-Mouth Disease Virus neutralizing antibodies production induced by pcDNA3 and Sindbis virus based plasmid encoding FMDV P1-2A3C3D in swine

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ABSTRACT

DNA vaccination against Foot-and-Mouth Disease Virus (FMDV) is an attractive and alternative strategy to the use of classical inactivated viral vaccines. The injection of a pcDNA3.1-based DNA vaccine encoding for FMDV P1-2A3C3D and GM-CSF proteins had previously been shown to induce the production of neutralizing antibodies against FMDV and partially protect swine against an experimental challenge. Based on the induction of FMDV humoral immune responses, the aim of the present study was to see if the Sindbis virus derived plasmid (pSINCP) backbone could advantageously replace pcDNA3.1 in DNA immunization against FMDV in swine. For this purpose, groups of 3 or 4 pigs received three injections by intramuscular route, intradermal route or an association of both routes, at 2–3 week intervals. The pcDNA3.1-based DNA vaccine was shown to induce the production of higher amounts of FMDV-neutralizing antibodies after intradermal injection. Intramuscular injection of the same vaccine, or intramuscular (IM) and/or intradermal (ID) injection of the pSINCP-based DNA vaccine resulted in a significantly lower induction of FMDV-neutralizing antibodies. In conclusion, the humoral immune response of a DNA vaccine encoding for FMDV P1-2A3C3D was not improved by the pSINCP backbone and was higher when the plasmids were injected by the intradermal route.

1. Introduction

Foot-and-Mouth Disease Virus (FMDV) is the etiological agent of an important disease of livestock. Foot-and-Mouth Disease (FMD) is highly contagious and affects cloven-hoofed animals, mostly cattle, swine, sheep and goats. FMDV belongs to the Aphthovirus genus of the Picornaviridae family (Rodrigo and Dopazo, 1995; Sobrino et al., 2001). The positive-strand RNA genome of about 8500 nucleotides length is enclosed within a protein capsid. The viral open reading frame (ORF) encodes a single polyprotein that is cleaved by viral proteases to yield different structural and non-structural proteins (Ryan et al., 1989).

Regular vaccination is one of the strategies employed to control disease propagation, and has resulted in eradication of the disease in some parts of the world (particularly Western Europe) (Sobrino et al., 2001). Efficient vaccination was achieved with adjuvanted chemically inactivated FMDV, which induced a consistent humoral response. Nevertheless, this kind of vaccination does have some disadvantages: (a) the production of FMDV requires high containment facilities and the risk of virus escape is still possible (Grubman and Baxt, 2004; The Veterinary Record, 2007), (b) inactivation of the virus may be incomplete in some cases (King et al., 1981), (c) the induced protection is short lasting (Cox et al., 2003) and (d) discriminating between vaccinated and infected animals can be a problem with some vaccines due to the lack of validated differentiation techniques. For all these reasons, alternative vaccination strategies such as the use of proteins, peptides, replicating vectors, attenuated strains and DNA vaccines have been investigated (Grubman and Baxt, 2004; Sobrino et al., 2001). Among these, DNA vaccination presents several advantages. In fact, the production of DNA vaccines is easy and safe, long-term storage of the vaccine is possible, constructs encoding fusion proteins can be developed and DNA vaccines can serve as marker vaccines. This technology can also be used efficiently to create vaccines against emerging serotypes. Numerous trials have been carried out in this context to obtain an efficient DNA vaccine against FMDV, with various degrees of success. Plasmids encoding FMDV VP1 (Park et al., 2006; Xiao et al., 2007) or FMDV epitopes (Cedillo-Barrón et al., 2003; Chen and Shao, 2006; Du et al., 2007; Zhang et al., 2003) were tested. Three injections of a plasmid encoding the viral structural protein precursor P1-2A and the non-structural proteins 3C and 3D, together with a plasmid encoding GM-CSF, induced the production of FMDV-specific and

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neutralizing antibodies and partially protected pigs from an experimental FMDV infection (Cedillo-Barrón et al., 2001). It is apparent from these results that the efficiency of a DNA vaccine needs to be considerably improved. In fact, the number of injections required is too high for a vaccine that must be able to act rapidly in the case of an FMDV outbreak. Stronger FMDV-specific and neutralizing antibody responses were induced by increasing the amount of plasmids (Li et al., 2006), but not by co-injecting a plasmid encoding the B cell activating factor (BAFF) protein (Bergamin et al., 2007). Interestingly, two co-injections of the FMDV and GM-CSF constructs, both formulated with D,L-lactide-*co*-glycolide based particles, induced humoral and cytotoxic immune responses and protected 5 out of 5 sheep against a virulent FMDV challenge (Niborski et al., 2006).

A new generation of non-replicating plasmids derived from the Sindbis virus (pSINCP) was generated in 1996 (Dubensky et al., 1996). This approach involved the conversion of a self-replicating vector RNA (replicon) into a layered DNA-based expression system. The first layer includes a eukaryotic RNA polymerase II expression cassette that initiates nuclear transcription of an RNA which corresponds to the Sindbis virus vector replicon. After transport of this RNA from the nucleus to the cytoplasm, the second layer proceeds according to the Sindbis virus replication cycle and results in expression of the heterologous gene. For example, increased efficacies of anti-herpes simplex virus (Hariharan et al., 1998) and anti-*Mycobacterium tuberculosis* (Kirman et al., 2003) vaccinations were obtained with these plasmids. Furthermore, we demonstrated that a single injection of 13 μg of pSINCP encoding Pseudorabies virus (PrV) glycoproteins gB, gC and gD, i.e., 25 times less than for pcDNA3, efficiently protected pigs against a highly virulent experimental PrV challenge (Dory et al., 2005).

The aim of the present study was to compare the level of immunization of pigs injected with the FMDV P1-2A3C3D construct cloned either in pcDNA3.1 or in pSINCP. Either pcDNA3.1/GM-CSF or pSINCP/GM-CSF was used as adjuvant in the corresponding groups. Two quantities of FMDV construct-encoding plasmids were tested: the one originally used by Cedillo-Barrón et al. (2001) and one 25 times smaller, as used in our previous PrV study (Dory et al., 2005). In a second part of the study, the efficacies of pig immunization by intramuscular (IM) or intradermal (ID) injection were compared.

2. Materials and methods

2.1. Plasmids

pcDNA3.1 plasmid encoding the FMDV O_1K P1-2A3C3D sequence (Cedillo-Barrón et al., 2001), and 9829 bp in length, was kindly provided by Paul Barnett (IAH, Pirbright, UK). The P1-2A3C3D cassette was extracted by a blunt *Pme* I digestion and inserted into the dephosphorylated blunt *Pml* I site of the pSINCP plasmid (kindly provided by John Polo, Chiron Corporation, USA) (Fig. 1). pSINCP encoding FMDV O_1K P1-2A3C3D sequences was selected by endonuclease restrictions, PCR and sequencing (not shown). The resulting plasmid was 16,306 bp long. 4.5×10^5 porcine kidney-derived PK15 cells per well were incubated for 24 h at 37 °C in a 6-well plate. These 70–80% confluent cells were then transfected either with 2 μg of pcDNA3.1/P1-2A3C3D, pSINCP/P1-2A3C3D, empty-pcDNA3.1 or empty-pSINCP by using lipofectamine plus transfection reagent (Invitrogen, Gaithersburg, MD) according to the Manufacturer's instructions. Forty-eight hours later, the expression was determined by immunostaining with a mouse anti-VP1 monoclonal antibody B2 (kindly provided by Emiliana Brocchi, IZS, Brescia, Italy) (Cedillo-Barrón et al., 2001) followed by incubation with a HRP-conjugated goat anti-mouse antibody and the peroxidase AEC substrate (Serotec Ltd., Oxford, UK) (Fig. 1). pCDNA3.1 plasmid encoding porcine GM-CSF (Dufour et al., 2000; Somasundaram et al., 1999) was kindly provided by François Lefèvre (INRA, Jouy-en-Josas, France). The GM-CSF cassette was extracted by *Apa* I and *Not* I digestion and inserted into the dephosphorylated pSINCP plasmid digested with the same enzymes (Fig. 2A). pSINCP encoding porcine GM-CSF was characterized by endonuclease restrictions and sequencing (not shown). Porcine PK15 cells were transfected with 2 μg of pcDNA3.1/GM-CSF, pSINCP/GM-CSF, empty-pcDNA3.1 or empty-pSINCP as described above. Forty-eight hours later, supernatants of each cell culture were collected and evaluated for GM-CSF activity (Fig. 2B). This was done in vitro on TF-1 cells as previously described (Loizel et al., 2005). In fact, these cells only grow in the presence of GM-CSF. Briefly, TF-1 cells were incubated for 16 h with different concentrations of recombinant porcine GM-CSF (0–10 ng/ml) (R&D Systems, Minneapolis, MN) or with the supernatants collected above (final volume: 100 μl). Each culture condition was prepared in triplicate. Twenty microlitres

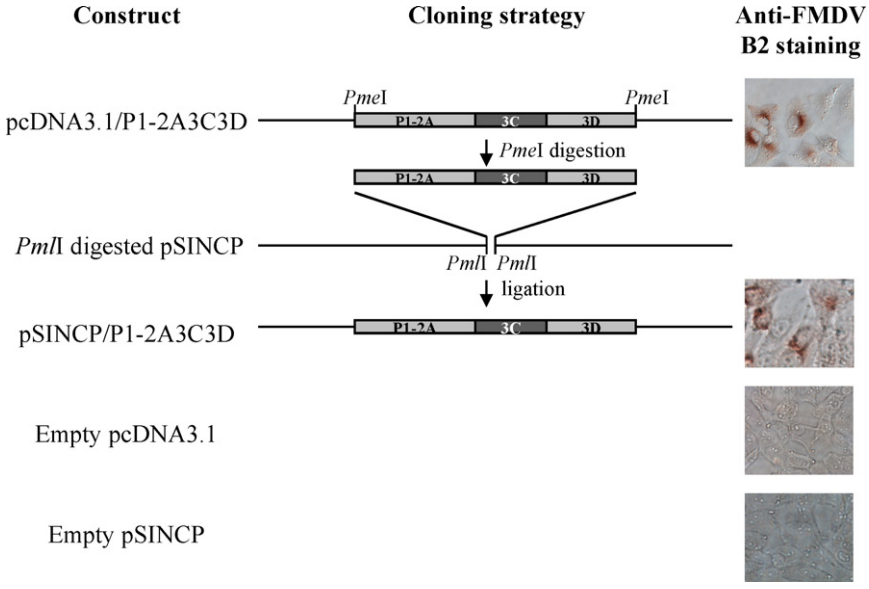

Fig. 1. Construction and characterization of the pSINCP/P1-2A3C3D construct. pSINCP/P1-2A3C3D was constructed as described in Section 2. Porcine PK15 cells transfected with pcDNA3.1/P1-2A3C3D, pSINCP/P1-2A3C3D, empty-pSINCP or non-transfected cells were stained with anti-VP1 monoclonal antibody B2 24 h after transfection.

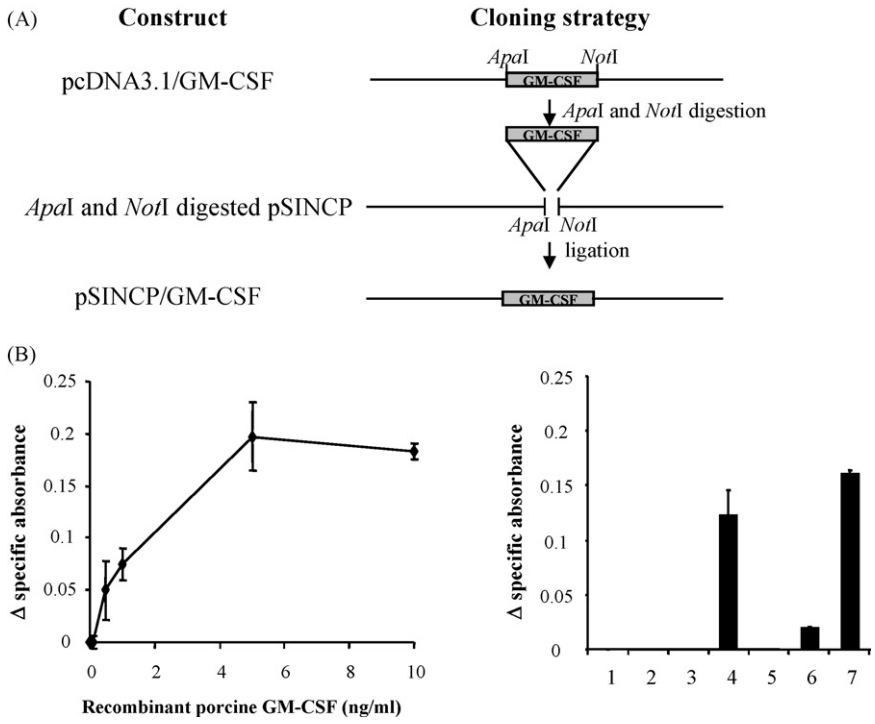

Fig. 2. Construction and characterization of the pSINCP/GM-CSF construct. (A) pSINCP/GM-CSF was constructed as described in Section 2. (B) *Left*: proliferation of TF-1 cells induced by increased concentrations of recombinant GM-CSF. *Right*: proliferation of TF-1 cells induced by incubation with supernatants of (1) non-transfected PK15 cells, (2) PK15 cells transfected with empty-pcDNA3.1, (3) PK15 cells transfected with pcDNA3.1/GM-CSF (1:10 diluted supernatant), (4) PK15 cells transfected with pcDNA3.1/GM-CSF (undiluted supernatant), (5) PK15 cells transfected with empty-pSINCP, (6) PK15 cells transfected with pSINCP/GM-CSF (1:10 diluted supernatant) and (7) PK15 cells transfected with pSINCP/GM-CSF (undiluted supernatant). Results shown are the mean ± SD of triplicates.

of Alamar Blue dye (Biosource International, Camarillo, CA) were then added. After 6 h incubation, the optical densities (OD) of the cultures were read at 600 nm (original oxidized form of the dye) and at 570 nm (reduced form). The specific absorbance of each culture corresponded to the difference between 600 and 570 nm OD. Proliferation of the TF-1 cells was represented by the difference between the specific absorbance of cells incubated with rGM-CSF or PK15 supernatants and that of non-stimulated cells (= Δ specific absorbance).

These plasmids, or empty-pcDNA3 or pSINCP plasmids were introduced into *Escherichia coli* XL-1 blue strain, amplified and purified using the EndoFree plasmid Mega kit (Qiagen, Hilden, Germany) according to the Manufacturer's instructions.

2.2. Animal experiments

Two experiments were performed in pigs. In the first, 7 groups of 3 unvaccinated large white pigs obtained from an air-filtered farm were housed and treated in accordance with the regulations of the local veterinary office (Direction des Services Vétérinaires des Côtes d'Armor, France). The pigs were intramuscularly injected in the neck 3 times at 2-week intervals with 2 ml of plasmids DNA using 0.8 mm × 40 mm needles. The first injection was administered when the pigs were 7 weeks old.

At each injection time, 600 μg or 24 μg of pcDNA3.1/P1-2A3C3D were co-injected with 200 μg of pcDNA3.1/GM-CSF in group 1 or group 2, respectively. Since pSINCP/P1-2A3C3D is 1.7 times longer than pcDNA3.1/P1-2A3C3D, groups 3 and 4 were co-injected with 1020 μg or 40 μg of pSINCP/P1-2A3C3D and 340 μg pSINCP/GM-CSF at each injection time, respectively. Group 5 was co-injected with 600 μg of empty-pcDNA3.1 and 200 μg of pcDNA3.1/GM-CSF. Group 6 was co-injected with 1020 μg empty-pSINCP and 340 μg pSINCP/GM-CSF. Group 7 was not injected throughout the assay.

In the second assay, 8 groups of 4 specific pathogen-free pigs were injected by IM and/or ID routes 3 times at 3-week intervals. The first injection was administered when the pigs were 7 weeks old. At each injection time, pigs were injected either with a total 600 μg of pcDNA3.1/P1-2A3C3D + 200 μg of pcDNA3.1/GM-CSF or 1020 μg of pSINCP/P1-2A3C3D + 340 μg pSINCP/GM-CSF. The ID injection was done in the dorsal surface of both ears using 0.45 mm × 12 mm needles. It was controlled by (i) the parallel position of the needle and the ear surface, (ii) the high pressure applied on the syringe to inject the solutions and (iii) the transient generation of white spots. As in the first assay, groups 1 and 2 received an IM injection (1 × 2 ml) of the pcDNA3.1-based or pSINCP-based DNA vaccine, respectively. Groups 3 and 4 received either an IM injection (half the DNA quantity, 1 ml in each side of the animal) and an ID injection (half the DNA quantity, 0.25 ml on the top of each ear) of the pcDNA3.1-based or pSINCP-based DNA vaccine, respectively. Groups 5 and 6 were ID injected with the pcDNA3.1-based or pSINCP-based DNA vaccine, respectively (0.25 ml on the top of each ear). Two other groups were injected either with 600 μg of pcDNA3.1/P1-2A3C3D + 200 μg of pcDNA3.1/GM-CSF or 1020 μg of pSINCP/P1-2A3C3D + 340 μg of pSINCP/GM-CSF by both routes (IM + ID), respectively.

The pigs were observed for any adverse reaction after injection. Body temperature was measured 4 h after injection then daily. Relative daily weight gains were determined (Stellmann et al., 1989) for each pig. Finally, the pigs were sacrificed at the end of the assay and the injected region and other organs were examined to see whether the injection of plasmids produced lesions on these organs.

2.3. Determination of FMDV-specific serum antibodies

Anti-FMDV antibodies were first measured in pig sera using a commercial test: Ceditest FMDV type O (Cedi Diagnostics B.V., Lelystad, The Netherlands) which is a blocking Elisa (Chénard et

al., 2003). Positive serums are those presenting 50% or more inhibition compared to a high positive reference serum. In order to detect very low levels of antibodies, an Elisa test was set up as follows: 96-well, flat-bottomed plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with a rabbit anti O1 BFS antiserum diluted in carbonate/bicarbonate buffer pH 9.6 (Sigma, St. Louis, MO). Plates were blocked with PBS-tween20 0.05% buffer supplemented with 10% bovine serum and 5% rabbit serum. The plates were then incubated with FMDV antigen (O1 BFS) for 1 h at 37 °C. After washings, samples of pig sera diluted 1/40 in blocking buffer were added in duplicate to an antigen-coated well and to a control well. After washings and incubation with a HRP (Horse Radish Peroxidase) conjugated anti-swine serum, the reaction was revealed with an *ortho*-phenylene-diamine (OPD) solution (Sigma). Results were expressed as the difference in measured OD (optical density) between the antigen-coated and the control wells. A serum is considered as positive if the difference of OD is superior to 0.20.

2.4. FMDV-neutralizing antibodies

Neutralizing antibody assays were carried out in 96 wells as described in the OIE ("Office International des Epizooties") Manual of Standards (OIE, 2000). Serial dilutions of sera were performed in duplicate and 50 μl of each were added for I h to 50 μl of 100 CCID 50 of FMDV O1 BFS strain. Cell suspension was then added to each well and the plates were incubated at 37 °C for 3 days. The cells were fixed with formalin and stained with methylene blue. Titres were expressed as the last serum dilution that inhibited viral replication in 50% of the wells.

2.5. Statistical analyses

The data were analyzed using the nonparametric Mann–Whitney test (Mann and Whitney, 1947) included in the Systat 9 software (Systat Software, Inc., Point Richmond, CA). This test was used as the generated data were few in number, did not present a normal distribution and consisted of unpaired quantitative data.

The limit of significance was 0.05 for all comparisons.

3. Results

In vitro assays showed that each FMDV P1-2A3C3D construct and each GM-CSF construct were effectively expressed in the porcine cell line PK15 (see Figs. 1 and 2 and Section 2). PK15 cells transfected with pSINCP/GM-CSF produced more GM-CSF than cells transfected with pcDNA3.1/GM-CSF (Fig. 2). The immune potentials of the FMDV constructs were assessed in swine. Serum samples

Table 1
Induction of anti-FMDV-specific and neutralizing antibodies after intramuscular injection of different constructs.

Group	Pig #		Day 0	Day 7 *Inj1 + 7 d*	Day 14 *Inj1 + 14 d*	Day 21 *Inj2 + 7 d*	Day 28 *Inj2 + 14 d*	Day 35 *Inj3 + 7 d*	Day 42 *Inj3 + 14 d*
pcDNA3.1/P1-2A3C3D 600 μg	1.1	OD	0.02	0.03	0.08	0.00	0.00	0.00	0.00
		NAb titre	0	0	**4**	0	0	0	0
	1.2	OD	0.02	0.00	0.00	0.05	0.15	0.00	0.00
		NAb titre	0	0	**3**	**2**	0	**2**	**2**
	1.3	OD	0.00	0.06	0.04	0.09	0.00	0.00	0.02
		NAb titre	0	0	0	**3**	**2**	**3**	**2**
pcDNA3.1/P1-2A3C3D 24 μg	1.4	OD	0.00	0.00	0.00	0.00	0.07	0.00	0.00
		NAb titre	0	0	**2**	**3**	**2**	0	0
	1.5	OD	0.06	0.07	0.08	0.04	0.04	0.11	0.00
		NAb titre	0	0	0	0	0	0	0
	1.6	OD	0.07	0.00	0.06	0.04	0.02	0.00	0.00
		NAb titre	0	0	0	0	0	0	0
pSINCP/P1-2A3C3D 1020 μg	1.7	OD	0.00	0.07	0.00	0.05	0.00	**0.68**	**0.37**
		NAb titre	0	0	0	0	**3**	0	3
	1.8	OD	0.06	0.12	0.00	**0.48**	**0.28**	**1.02**	**1.00**
		NAb titre	0	0	0	0	0	0	0
	1.9	OD	0.10	0.08	0.10	**0.21**	0.15	0.06	**0.37**
		NAb titre	0	0	0	0	**4**	**2**	**6**
pSINCP/P1-2A3C3D 40 μg	1.10	OD	0.03	0.04	0.00	0.00	0.06	0.04	0.04
		NAb titre	0	0	0	0	0	0	0
	1.11	OD	0.00	0.05	0.02	0.06	0.00	0.00	0.00
		NAb titre	0	0	0	0	0	0	0
	1.12	OD	0.10	0.08	0.07	0.10	0.10	0.02	0.00
		NAb titre	0	0	**3**	**3**	**4**	**3**	**3**
Empty-pcDNA3.1 600 μg	1.13	OD	0.01	0.00	0.00	0.00	0.04	0.03	0.00
		NAb titre	0	0	0	0	0	0	0
	1.14	OD	0.00	0.09	0.00	0.06	0.00	0.05	0.00
		NAb titre	0	0	0	0	0	0	0
	1.15	OD	0.14	0.04	0.10	0.09	0.08	0.00	0.00
		NAb titre	0	0	0	0	0	0	0
Empty-pSINCP 1020 μg	1.16	OD	0.02	0.00	0.00	0.00	0.00	0.00	0.00
		NAb titre	0	0	0	0	0	0	0
	1.17	OD	0.06	0.00	0.04	0.08	0.05	0.03	0.00
		NAb titre	0	0	0	0	0	0	0
	1.18	OD	0.00	0.00	0.06	0.02	0.00	0.00	0.00
		NAb titre	0	0	0	0	0	0	0

Anti-FMDV-specific and neutralizing antibodies titres were determined after injections of 600 μg or 24 μg of pcDNA3.1/P1-2A3C3D, 1020, 40 μg of pSINCP/P1-2A3C3D, 600 μg of empty-pcDNA3.1 or 1020 μg of empty-pSINCP. Specific antibodies titres were determined by ELISA as described in Section 2. The optical densities are shown. Serums with optical densities superior to 0.20 are considered as positive. Anti-FMDV-neutralizing antibodies titres were determined as described in Section 2.
In bold are indicated samples considered as positives for the Elisa test (OD > 0.20) or with a NAb titre > 0.
Inj: injection; *d: days.*

from all pigs were analyzed by ELISA and viral neutralization assays for FMDV-specific and FMDV-neutralizing antibody responses.

First, the capacity of a plasmid derived from the Sindbis virus as backbone to enhance the induction of humoral immune response or to significantly reduce the quantity of plasmids needed for the FMDV immunization was evaluated. An assay was performed to compare 2 different quantities of each of the 2 plasmid backbones (pcDNA3.1 and pSINCP). In the case of pcDNA3.1, 600 μg of plasmids were injected in one group as in previous studies (Cedillo-Barrón et al., 2001) and 25 times fewer plasmids, i.e., 24 μg, were injected in another group in accordance with the results with PrV DNA vaccination (Dory et al., 2005). Since the pSINCP-based construct is 1.7 times longer than the pcDNA3.1-based one, 1020 μg or 40 μg of pSINCP/P1-2A3C3D were injected in two other groups in order to use the same number of copies of molecules. The plasmids were injected by IM route since this was the one used for the PrV-pSINCP study (Dory et al., 2005) and in many FMDV DNA vaccination studies (Guo et al., 2005, 2004; Wong et al., 2002; Zhang et al., 2003). No fever, adverse reaction or modification of the daily weight gains was observed in any group after any injection. The results show that these vaccines were well tolerated by the animals. The production of specific antibodies against FMDV was assessed by ELISA. No antibodies were detected in the groups injected with the smaller quantities of FMDV P1-2A3C3D encoding plasmids or in the control groups (not shown). No antibodies were detected in pigs injected with 600 μg of pcDNA3.1/P1-2A3C3D + pcDNA3.1/GM-CSF (Table 1). Seven days after the second injection, low levels of antibodies were detected in the group injected with 1020 μg of pSINCP/P1-2A3C3D + pSINCP/GMCSF and antibody production was detected in all 3 pigs 14 days after the last injection. Extremely low and non-significant levels of neutralizing antibodies were detected in the groups injected with high quantities of FMDV P1-2A3C3D encoding plasmids. Interestingly, neutralizing antibodies were detected transiently or until the end of the assay in 1 out of 3 pigs in the groups injected with 24 μg of pcDNA3.1/P1-2A3C3D + pcDNA3.1/GM-CSF or 40 μg of pSINCP/P1-2A3C3D + pSINCP/GM-CSF, respectively. Nevertheless, collectively these data show that the pSINCP and the pcDNA3.1-based DNA vaccine injected via the IM route induced a low production of FMDV-specific and neutralizing antibodies. Except for the production of specific antibodies, where the pSINCP-based DNA vaccine induced stronger responses, there were no differences between the 2 kinds of plasmids. No lesions were apparent in any of the organs observed during necropsy of the animals at the end of the assay.

In a second assay, the influence of the route of administration of the DNA vaccines was studied. In this context, and based on several papers published with this FMDV P1-2A3C3D construct (Cedillo-Barrón et al., 2001; Li et al., 2006; Niborski et al., 2006), ID and IM injections were compared. As no specific antibodies were detected in the group injected with 40 μg of pSINCP/P1-2A3C3D, whereas all pigs in the group injected with 1020 μg of plasmids were positive, the second study was limited to the injection of the largest

Table 2
Induction of anti-FMDV-specific antibodies after intramuscular and/or intradermal injection of different constructs.

Group	Pig #	Day 0	Day 14 *Inj1 + 14 d*	Day 21 *Inj1 + 21 d*	Day 28 *Inj2 + 7 d*	Day 35 *Inj2 + 14 d*	Day 42 *Inj2 + 21 d*	Day 49 *Inj3 + 7 d*	Day 56 *Inj3 + 14 d*	Day 63 *Inj3 + 21 d*
pcDNA3.1/P1-2A3C3D	2.1	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
IM	2.2	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
	2.3	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
	2.4	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
pSINCP/P1-2A3C3D	2.5	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
IM	2.6	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
	2.7	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
	2.8	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
pcDNA3.1/P1-2A3C3D	2.9	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	**52.5**	<50.0
IM + ID	2.10	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	**57.4**	**54.4**
	2.11	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	**54.4**	<50.0
	2.12	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	**57.0**	**55.0**
pSINCP/P1-2A3C3D	2.13	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	**63.4**	**52.3**
IM + ID	2.14	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	**50.0**	<50.0
	2.15	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
	2.16	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
pcDNA3.1/P1-2A3C3D	2.17	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	**52.9**	<50.0
ID	2.18	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	**50.0**	**68.2**	**59.6**
	2.19	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	**50.0**	<50.0
	2.20	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	**50.0**
pSINCP/P1-2A3C3D	2.21	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
ID	2.22	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
	2.23	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
	2.24	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
Empty-pcDNA3.1	2.25	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
IM + ID	2.26	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
	2.27	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
	2.28	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
Empty-pSINCP	2.29	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
IM + ID	2.30	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
	2.31	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0
	2.32	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0	<50.0

Anti-FMDV-specific antibodies productions were determined after IM, ID or IM + ID injections of pcDNA3.1/P1-2A3C3D or pSINCP/P1-2A3C3D, or after IM + ID injections of empty-pcDNA3.1 or empty-pSINCP. The level of these antibodies was measured using a blocking ELISA and are indicated as % of inhibition of a positive control reference serum. Positive serums (Pos) are those presenting 50% or more inhibition.
In bold are inidcated positive samples (% of inhibition ≥ 50).
Inj: injection; *d: days*.

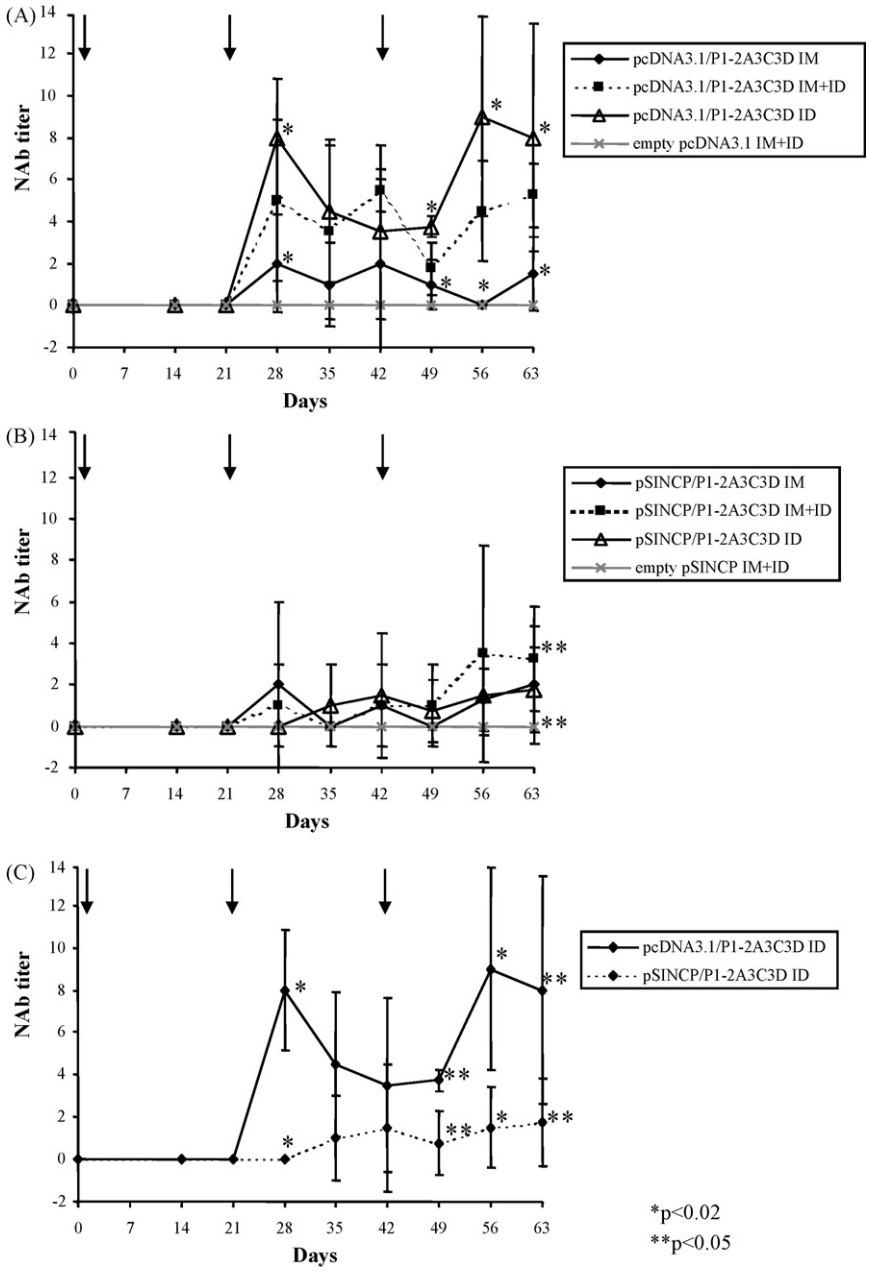

Fig. 3. Induction of anti-FMDV-neutralizing antibodies after intramuscular and/or intradermal injection of different constructs. (A) Neutralizing antibodies titres after IM, ID or IM + ID injections of the pcDNA3.1-based DNA vaccine. Mean titres ± SD are shown. (B) Neutralizing antibodies titres after IM, ID or IM + ID injections of the pSINCP-based DNA vaccine. Mean titres ± SD are shown. (C) Neutralizing antibodies titres after ID injections of pcDNA3.1- or pSINCP-based DNA vaccine. Mean titres ± SD are shown. $^{*}p < 0.02$; $^{**}p < 0.05$.

quantity of plasmids used above. Plasmids encoding GM-CSF were co-injected in all pigs. As in the first assay, no fever, adverse reaction or modification of the daily weight gains was observed after any injection in any of the animal groups. No FMDV-specific and neutralizing antibodies were detected in the groups injected with empty-pcDNA3, empty-pSINCP or in non-injected pigs (data not shown). As in the first assay, no or few antibodies were detected in the group injected by IM route (Table 2 and Fig. 3). Three out of 4 pigs developed antibodies against FMDV 14 days after the third ID injection of pcDNA3.1/P1-2A3C3D plasmid (Table 2). The 4th pig was found positive 1 week later. All pigs injected with the pcDNA3.1-based DNA vaccine by ID + IM routes were found positive 2 weeks after the third injection. For the groups injected with pSINCP/P1-2A3C3D, only 2 out of 4 pigs were detected positive in the IM + ID injected group. No positive pigs were detected in the IM or ID injected groups.

As in the first assay, neutralizing antibodies against FMDV were first detected after the second injection (Fig. 3). IM injections resulted in the production of low neutralizing antibodies titres with both plasmid backbones (Fig. 3A and B). ID injection resulted in a significant increase of the production of neutralizing antibodies when pcDNA3.1/P1-2A3C3D was used (Fig. 3A) but not when pSINCP/P1-2A3C3D was used (Fig. 3B). The pcDNA3.1-based DNA vaccine induced a significantly higher production of neutralizing antibodies than the pSINCP-based vaccine (Fig. 3C). On the other hand, IM + ID injection did not result in a significant increase of the production of neutralizing antibodies in either case, compared to the group injected by IM route only. No lesions were apparent in

any of the organs observed during necropsy of the animals at the end of the assay.

4. Discussion

Vaccination is one of the most important strategies used to control FMDV infection (Grubman and Baxt, 2004; Sobrino et al., 2001). Although vaccination with inactivated viruses has been shown to be efficient, it is associated with several problems related to safety and to discrimination between vaccinated and infected animals. These disadvantages might be overcome by using DNA vaccination. Several strategies designed to increase the efficacy of DNA vaccines have been evaluated. For example, GM-CSF (Cedillo-Barrón et al., 2001), IL-18 (Mingxiao et al., 2007), C3d (Fan et al., 2007), IL-2 (Wong et al., 2002) or electroporation (Kim et al., 2006) were described as promising adjuvants or strategies to increase vaccination efficacy, whereas several other strategies failed (Bergamin et al., 2007; Guo et al., 2004). Despite this progress, and as is the case for the FMDV P1-2A3C3D construct used here, 3 injections of plasmids are often needed (Cedillo-Barrón et al., 2001; Li et al., 2006; Xiao et al., 2007). Under these conditions, DNA vaccination is not expected to be useful to protect pigs in the case of a FMDV outbreak. Other strategies, that generated promising results in other models, should therefore be tested for FMDV DNA vaccination. This is the case for a new generation of non-replicative plasmids derived from the Sindbis virus that amplifies transcription of the replicons encoded by this plasmid (Dubensky et al., 1996). Use of this plasmid was able, for example, to enhance protection against a lethal herpes simplex virus infection (Hariharan et al., 1998) or to decrease the quantity of plasmids needed for immunization against *Mycobacterium tuberculosis* (Kirman et al., 2003) or a lethal PRV (Dory et al., 2005) infection. The potential beneficial effect of this kind of strategy in DNA vaccination against FMDV has not been examined before, and was the aim of the present study. The FMDV P1-2A3C3D cassette was inserted into the pSINCP plasmid and the ability of this construct to induce the production of FMDV antibodies was compared with that of the pcDNA3.1-based DNA vaccine. As in the original study (Cedillo-Barrón et al., 2001; Li et al., 2006), a plasmid encoding porcine GM-CSF was co-injected. As in previous studies using pSINCP-based DNA vaccines (Hariharan et al., 1998; Leitner et al., 2000), the DNA vaccines were injected by the IM route in an initial study. IM injection of the pcDNA3.1-based DNA vaccine led to no or a low production of FMDV-specific or neutralizing antibodies respectively. The production of neutralizing antibodies in particular was below that obtained by Cedillo-Barrón et al. (2001) with similar quantities of the same pcDNA3.1-based construct, when the plasmids were injected by the IM + ID routes. IM injection of the pSINCP-based DNA vaccine induced low or non-significant amounts of neutralizing antibodies. For each DNA vaccine, a 25-fold decrease of the quantity of plasmids resulted in a very low induction of FMDV antibodies. For some samples, neutralizing antibodies were found at a titre lower than 6, whereas no specific antibodies were detected by Elisa. An explanation of these discrepancies could be that the level of neutralizing antibodies is too low, and maybe non-significant. In fact, a special attention has to be made when interpreting low neutralization titres (Chénard et al., 2003). Furthermore, for some other samples, specific but no neutralising antibodies were detected. All the discrepancies observed could be due to the fact that the Elisa and the virus neutralization test are detecting different populations of antibodies (Chénard et al., 2003). It can be hypothesized from these results that the IM route of injection is not the best one to induce FMDV humoral immune responses. Thus a second assay using IM and/or ID routes of injection was performed. ID or ID + IM injections of the pSINCP-based DNA vaccine again induced a low production of FMDV antibodies. In contrast, ID injection of the pcDNA3.1-based DNA vaccine induced the production of FMDV-specific antibodies in 4 out of 4 pigs and significantly higher titres of neutralizing antibodies than the IM injection, as early as 7 days after the second injection of plasmids. The ID route was shown here to be the main one to induce production of FMDV-specific neutralizing antibodies. DNA vaccines applied to the surface of the skin have been described to mainly induce the production of a Th2 immune response in different disease models (Hahn et al., 2004, Zhu et al., 2004). This may be due to the presence of Langerhans or other dentritic cells in the dermis (Leitner et al., 1999; Peachman et al., 2003; Raz et al., 1994). Furthermore, the fact that the concentration of the injected plasmids was 4 times higher in the ID group (1200 μg/ml) than in the IM one (300 μg/ml) may also explain the observed discrepancies. Finally, due to the properties of the pSINCP plasmid (Dubensky et al., 1996), GM-CSF can potentially be produced at a high toxic concentration (Serafini et al., 2004) in animals injected with the pSINCP/GM-CSF construct. The results presented here also suggest that the dermal dentritic cells are more efficiently transfected with pcDNA3.1-based plasmids than with pSINCP-based plasmids. This might be due to the larger size of the pSINCP-based DNA vaccine (16306 bp vs 9829 bp for the pcDNA3.1-based construct) (Yin et al., 2005). Some neutralizing antibodies were detected after the IM injection. It is hypothesized that transfected myocytes (Leitner et al., 1999) may also indirectly induce the production of antibodies or that non-muscular cells might have been transfected after blood transport of some plasmid molecules (Gravier et al., 2007).

At this stage, no conclusions can be drawn about the protective ability of the pSINCP/P1-2A3C3D based DNA vaccine. In fact, there are descriptions in the literature of protection in the presence of low titres (Sobrino et al., 2001) or even in the absence (Borrego et al., 2006) of FMDV-neutralizing antibodies. The protective efficacy of this DNA vaccine can only be evaluated experimentally. Nevertheless, with the same FMDV antigens used, the level of neutralizing antibodies was significantly much lower with the pSINCP-based DNA vaccine than with the pcDNA3.1-based one previously shown to induce partial protection against FMDV infection (Cedillo-Barrón et al., 2001). Therefore, the possibility that the pSINCP-based vaccine confers partial (or no) protection is strong. It was therefore decided not to perform a FMDV challenge, which is the sole way of measuring the strength of a FMDV vaccine, in order to prevent the pigs from useless suffering. Furthermore, ID injection of the pcDNA3.1-based DNA vaccine was shown here to be the best way of inducing the production of FMDV-specific neutralizing antibodies. It would be very interesting to evaluate the protective potential of this DNA vaccine injected by ID route after the second, or even first, injection of plasmids.

In conclusion and contrary to our initial hypothesis, the pSINCP plasmid was shown to be unable to enhance the production of FMDV-neutralizing antibodies of the DNA vaccine against FMDV encoding P1-2A3C3D. Furthermore, the route of injection of the plasmids was shown to be essential for the induction of immune responses as the ID route showed higher production of neutralizing antibodies than the intramuscular one.

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